PCT

WORLD INTELLECTUAL
Internati

INTERNATIONAL APPLICATION PUBLISHED UN

| (51) International Patent Classification 6 : C07K 14/57, 16/24 | A2 | (11) International Publication Number: WO 96/03435 |
| | | (43) International Publication Date: 8 February 1996 (08.02.96) |

(21) International Application Number: PCT/GB95/01731

(22) International Filing Date: 21 July 1995 (21.07.95)

(30) Priority Data:
9414752.7   21 July 1994 (21.07.94)   GB

(71) Applicant *(for all designated States except US):* Q-ONE BIOTECH LIMITED [GB/GB]; Todd Campus, West of Scotland Science Park, Glasgow G20 0XA (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only):* ONIONS, David, Edward [GB/GB]; Windyhill Farm, Barochan Road, Houston, Renfrewshire PA6 7AA (GB). ARGYLL, David [GB/GB]; 47 Maxwell Avenue, Westerton, Bearsden, Glasgow G61 1PA (GB). LEES, Gillian, Margaret [GB/GB]; 5 Kirkuace Drive, Newton Mearns, Glasgow G77 5HD (GB).

(74) Agents: McCALLUM, William, Potter et al.; Cruikshank & Fairweather, 19 Royal Exchange Square, Glasgow G1 3AE (GB).

(81) Designated States: AU, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: FELINE γ-INTERFERON

```
CTACTGATTTCAACTTCTTTGGCCTAACTCTCCGAAACGATGAATTACACAAGTTTTATT
          *   L  S  E  T  M  N  Y  T  S  F  I

TTCGCTTTCCAGCTTTGCATAATTTTGTGTTCTTCTGGTTATTACTGTCAGGCCATGTTT
F  A  F  Q  L  C  I  I  L  C  S  S  G  Y  Y  C  Q  A  M  F

TTTAAAGAAATAGAAGAGCTAATGGGATATTTTAATGCAAGTAATCCAGATGTAGCAGAT
F  K  E  I  E  E  L  M  G  Y  F  N  A  S  N  P  D  V  A  D

GGTGGGTCGCTTTTCGTAGACATTTTGAAGAACTGGAAAGAGGAGAGTGATAAAACAATA
G  G  S  L  F  V  D  I  L  K  N  W  K  E  E  S  D  K  T  I

ATTCAAAGCCAAATTGTCTCCTTCTACCTGAAAATGTTTGAAAACCTGAAAGATGATGAC
I  Q  S  Q  I  V  S  F  Y  L  K  M  F  E  N  L  K  D  D  D

CAGCGCATTCAAAGGAGCATGGACACCATCAAGGAAGACATGCTTGATAAGTTGTTAAAT
Q  R  I  Q  R  S  M  D  T  I  K  E  D  M  L  D  K  L  L  N

ACCAGCTCCAGTAAACGGGATGACTTCCTCAAGCTGATTCAAATCCCTGTGAATGATCTG
T  S  S  S  K  R  D  D  F  L  K  L  I  Q  I  P  V  N  D  L

CAGGTCCAGCGCAAAGCAATAAATGAACTCTTCAAAGTGATGAATGATCTCTCACCAAGA
Q  V  Q  R  K  A  I  N  E  L  F  K  V  M  N  D  L  S  P  R

TCTAACCTGAGGAAGCGGAAAAAGGAGCCAGAATCTGTTTCGAGGCCGTAGAGCATCGAAA
S  N  L  R  K  R  K  R  S  Q  N  L  F  R  G  R  R  A  S  K

TAATGGTTGTCCTGCCTGCAATATTTG
     *
```

(57) Abstract

    The present invention provides a polynucleotide fragment encoding feline γ-interferon, a recombinant vector comprising such a polynucleotide fragment, a host cell containing said polynucleotide fragment or recombinant vector, a recombinant feline γ-interferon polypeptide, and pharmaceutical compositions comprising recombinant γ-interferon for use as a prophylactic and/or therapeutic agent and also as an adjuvant.

FELINE γ-INTERFERON

The present invention relates to feline γ-interferon, a polynucleotide fragment encoding feline γ-interferon, a recombinant vector comprising such a polynucleotide fragment, a host cell containing said polynucleotide fragment, or recombinant vector comprising said polynucleotide fragment, a feline γ-interferon polypeptide, antibodies immuno-reactive with said polypeptide and pharmaceutical compositions comprising recombinant feline γ-interferon for use as a prophylactic and/or therapeutic agent and also as an adjuvant in cats.

Cytokines are low molecular weight secreted proteins with immunomodulatory activity. The term applies to interferons, interleukins, lymphokines, monokines, colony stimulating factors and a number of growth factors (Balkwill and Burke 1989). Interferons are a class of cytokines which exhibit antiviral activity. Three types exist, alph (α), beta (ß) and gamma (γ) (Farrar and Schreiber 1993). The type I interferons, α and ß are related and bind to a common cell structure surface receptor, although the multiple forms of α and the single ß form may have different biological effects. Interferon gamma (IFNγ), also known as type II or immune interferon, is distinct from α and ß interferons at a genetic level. Like ß interferon it is encoded by a single gene. IFNγ is

2

produced by natural killer (NK) cells and by T lymphocytes (TH1 subset) in response to stimulation with antigen or mitogens. It possesses a range of biological effects of immunomodulatory or antiviral nature including enhancement of MHC class I and class II expression, suppression of the TH2 T lymphocyte response, activation of macrophages, enhancement of natural killer cell activity, and modulation of the synthesis and effect of a number of cytokines (Gray 1992). IFNγ has also exhibited adjuvanticity properties in some animal models (reviewed in Health and Playfair 1992).

Although the bovine and ovine IFNγs, which exhibit 93% amino acid identity, are cross-reactive (Radford et al, 1991), IFNγs tend to be highly species-specific in their biological activity. Thus investigation of the potential applications of IFNγ in a particular species generally requires production of recombinant IFNγ of that species. Since the goals of most interferon cloning operations are deduction of the amino acid sequence and production of recombinant IFN, most IFNγ sequences have been derived from mRNA rather than chromosomal DNA. The advent of the polymerase chain reaction has, however, facilitated the cloning of cytokine cDNAs and the nucleotide sequences of the genes or cDNAs encoding a number of mammalian cytokines to been determined.

The most extensive structural studies of IFNγ have been performed on the human cytokine. The human IFNγ gene is 6kbp in size and possesses 3 introns (Taya et al, 1982). The human IFNγ gene encodes a 166 amino acid pr cursor

possessing a 23 amino acid N-terminal signal sequence and two N-linked glycosylation sites. Functional IFNγ is homodimeric with the polypeptides associated in a non-covalent manner (Scahill et al, 1983). Several forms of human IFNγ exist due to post-translational C-terminal proteolytic cleavage and differential glycosylation (Rinderknecht et al, 1984). Regions of N-terminal and C-terminal sequence have been determined as being important in maintaining biological activity (Farrar and Schreiber, 1993).

Cytokine and anti-cytokine therapies such as synthesis of inhibitors, soluble cytokine receptors, receptor antagonists or anti-cytokine antibodies, are finding increased clinical application in human medicine (Mire-Sluis, 1993). Due to the lack of species cross-reactivity of many cytokines and the potential for antibody production against heterologous cytokines (Holmes, 1993), however, the ideal reagents in the cat will be substantially feline-specific.

The present invention provides a polynucleotide fragment, such as a DNA fragment, encoding feline γ-interferon. The invention further provides a recombinant feline γ-interferon polypeptide.

"Polynucleotide fragment" as used herein refers to a chain of nucleotides such as deoxyribose nucleic acid (DNA) sequences and transcription products thereof, such as RNA capable of giving rise to a feline γ-interferon protein or physiologically active fragments thereof. Thus, this term

4

includes double and single stranded DNA, and RNA sequences derived therefrom. The term excludes the whole naturally occurring genome.

Generally, the polynucleotide will be in isolated form substantially free of biological material with which the whole genome is normally associated _in vivo_.

In general, the term "polypeptide" refers to a chain or sequence of amino acids displaying a biological activity substantially similar to the biological activity of feline γ-interferon and does not refer to a specific length of the product as such. The polypeptide, if required, can be modified _in vivo_ and/or _in vitro_, for example by glycosylation, amidation, carboxylation, phosphorylation and/or post translational cleavage thus _inter alia_ peptides, oligopeptides and proteins are encompassed thereby.

The DNA fragment encoding feline γ-interferon can be amplified from feline γ-interferon cDNA obtained by way of reverse transcription of mRNA by polymerase chain reaction (PCR) techniques known in the art, using primers designed against conserved regions of γ-interferon coding sequences from a single species, or a number of other species. An amplified fragment containing the feline γ-interferon is depicted in Figure 1.

The DNA fragment of Figure 1 was shown to encode an open reading frame (ORF) of 171 amino acids. A comparison of the amino acid sequence against previously sequenced γ-interferons rev aled identities ranging from 68 % with

murine γ-interferon to 78% with human γ-interferon. The
two potential N-linked glycosylation sites exist at
positions conserved in bovine and pig γ-inteferon
precursors at amino acids 39-41 and 107-109 in the feline
precursor (data not shown). In common with the human γ-
interferon, feline γ-interferon has a predicted N-terminal
signal sequence and possesses no cysteine residues.

The present invention includes polynucleotide and
polypeptide sequences having at least 80 %, particularly at
least 90 % and especially at least 95 % similarity with the
sequences of Figure 1. "Similarity" refers to both
identical and conservative replacement of nucelotides or
amino acids, provided that the functionality of feline γ-
interferon is substantially unimpaired.

The skilled man will appreciate that it is possible to
genetically manipulate the gene or derivatives thereof, for
example to clone the gene by recombinant DNA techniques
generally known in the art and to express the polypeptides
encoded thereby in vitro or in vivo. Polynucleotide
fragments having the nucleotide sequence depicted in Figure
1 or derivatives thereof are preferably used for the
expression of the feline γ-interferon.

It will be understood that for the particular feline
γ-interferon polypeptide embraced herein, natural
variations can exist between individuals or between
species of the felis genus. These variations may be
demonstrated by (an) amino acid difference(s) in the
overall sequence or by deletions, substitutions,

insertions, inversions or additions of (an) amino acid(s)
in said sequence. All such derivatives showing active
feline γ-interferon physiological activity are included
within the scope of this invention. For example, for the
purposes of the present invention conservative replacements
may be made between amino acids within the following
groups:

(i)   Alanine, Serine, Threonine;

(ii)   Glutamic acid and Aspartic acid;

(iii)   Arginine and Lysine;

(iv)   Asparagine and Glutamine;

(v)   Isoleucine, Leucine, and Valine;

(vi)   Phenylalanine, Tyrosine and Tryptophan.

Moreover, recombinant DNA technology may be used to
prepare nucleic acid sequences encoding the various
derivatives outlined above.

As is well known in the art, the degeneracy of the
genetic code permits substitution of bases in a codon
resulting in a different codon which is still capable of
coding for the same amino acid, e.g. the codon for the
amino acid glutamic acid is both GAT and GAA. Consequently,
it is clear that for the expression of a polypeptide with
the amino acid sequence shown in Figure 1 or fragment
thereof use can be made of a derivative nucleic acid
sequence with such an alternative codon composition
differ nt from the nucleic acid sequence shown in said

Figure 1.

Furthermore, fragments derived from the feline

γ-interferon polypeptide or from the amino acid sequence

depicted in Figure 1 which still display feline

γ-interferon properties, or fragments derived from the

nucleotide sequence encoding the feline γ-interferon

polypeptide or derived from the nucleotide sequence

depicted in Figure 1 encoding fragments of said feline

γ-interferon polypeptides are also included in the present

invention.

All such modifications mentioned above resulting in

such derivatives of the feline γ-interferon polypeptide or

gene are covered by the present invention so long as the

characteristic feline γ-interferon biological properties

remain substantially unaffected.

The feline γ-interferon polynucleotide fragment of the

present invention is preferably linked to regulatory

control sequences. Such control sequences may comprise

promoters, operators, inducers, ribosome binding sites

terminators etc. Suitable control sequences for a given

host may be selected by those of ordinary skill in the art.

In particular, a feline γ-interferon control sequence can

be employed in a mammalian host.

A polynucleotide fragment according to the present

invention can be ligated to various expression controlling

polynucleotide sequences, resulting in a so-called

recombinant nucleic acid molecule. Thus the present

invention also includes an expression vector containing an expressible nucleic acid molecule. Said recombinant nucleic acid molecule can then be used for transformation of a suitable host. Such hybrid polynucleotide molecules are preferably derived from for example plasmids, or from nucleic acid sequences present in bacteriophages or viruses and are termed vector molecules.

Specific vectors which can be used to clone nucleic acid sequences according to the invention are known in the art (e.g. Rodriguez, R.L. and D.T. Denhardt, edit., Vectors: A survey of molecular cloning vectors and their uses, Butterworths, 1988).

A specific bacterial expression vector pHEX has been adapted for γ-interferon production (Reid, submitted for publication). The pHEX vector has a stretch of 6 histidine residues and a thrombin site downstream of the vector-specified ATG initiation codon. The 6 histidine residues provide crude purification of recombinant proteins using affinity chromatography. The DNA insertion site is positioned 3' to the thrombin site so that should biological activity of the feline γ-interferon produced be compromised by the vector-specified N-terminus, cleavage of extraneous amino-acid sequence from the interferon moiety is possible.

The methods to be used for the construction of a recombinant nucleic acid molecule according to the invention are known to those of ordinary skill in the art and are *inter alia* set forth in Sambrook, *et al*. (Molecular

9

Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1989).

The present invention also relates to a transformed cell containing the feline γ-interferon polynucleotide fragment in expressible form. "Transformation", as used herein, refers to the introduction of a heterologous polynucleotide fragment into a host cell, irrespective of the method used, for example direct uptake, transfection or transduction. The heterologous polynucleotide fragment may be maintained through autonomous replication or alternatively, may be integrated into the host genome. The recombinant nucleic acid molecules preferably are provided with appropriate control sequences compatible with the designated host which can regulate the expression of the inserted polynucleotide fragment, e.g. tetracycline responsive promoter, thymidine kinase promoter and SV-40 promoter.

Suitable hosts for the expression of recombinant nucleic acid molecules can be prokaryotic or eukaryotic in origin. The most widely used hosts for expression of recombinant nucleic acid molecules may be selected from bacteria, yeast, insect cells and mammalian cells.

Since the biological half-life and the degree of glycosylation of recombinant feline γ-interferon may be important for use in vivo, yeast and baculovirus systems are preferred. The yeast strain Pichia pastoris exhibits potential for high level expression of recombinant proteins (Clare et al,1991). The baculovirus system has been used

successfully in the production of type 1 interferons (Smith et al, 1983), and by the present invention for the production of feline γ-interferon.

The cloning and expression of recombinant feline γ-interferon also facilitates in producing reagents for the production of, for example, probes for in situ expression studies, production of anti-feline γ-interferon antibodies (particularly monoclonal antibodies) and evaluation of in vitro and in vivo biological activity of recombinant feline γ-interferon. The antibodies may be employed in diagnostic tests for γ-interferon.

The present invention further provides recombinant feline γ-interferon for the manufacture of reagents for use as prophylactic and/or therapeutic agents and also as an adjuvant in cats. In particular the invention provides pharmaceutical compositions comprising the recombinant γ-interferon together with a pharmaceutically acceptable carrier therefor.

A wide range of feline abnormalities such as disease states attributable to feline γ-interferon deficiencies or over abundance and physiological or immunological abnormalities could benefit from feline γ-interferon therapy and/or prophylaxis. Such disease states include cancer, endotoxaemia, parasitic and bacterial infections, wound therapy, auto-immune and inflammatory diseases, allergies and viral infections. Feline γ-interferon may also be applied for vaccine adjuvantation.

Cytokines such as interferons α, β and γ and tumour

necrosis factor, TNF, are reported as being capable of exhibiting direct antiviral activity in addition to their immunomodulatory properties (Ramsay et al, 1993). The recombinant feline γ-interferon can prove beneficial as a prophylactic agent to modulate recrudescence of viruses, such as feline herpesvirus (FHV), under conditions of stress and to reduce the clinical consequence of horizontal transmission of viral agents to in-contact animals. Herpes viruses and other viruses with complex genomes play an immunomodulatory role contributing to general or specific immunosuppression in the host. Administration of the feline γ-interferon can also serve to redirect the immune response such that more efficient virus clearance occurs. By way of example, administration of bovine IFNγ prior to experimental infection of cattle with bovine herpesvirus-1 (BHV-1) has been known to reduces virus-induced suppression of cell-mediated cytotoxicity (Bielefeldt Ohmann and Babiuk, 1984).

As a consequence of the pleiotropic nature of the cytokine system, administration of the recombinant feline γ-interferon may have consequences on the expression and biological effect of other cytokines. Thus feline γ-interferon can be used as a tool to study these effects.

Non-specific stimulation of the immune response by adjuvant formulations is often beneficial in cases of prophylactic or therapeutic vaccination. However, in particular instances a more defined stimulation of the immune response using feline γ-interferon as an adjuvant or

12

co-adjuvant can be more effective.   Such a stimulation

could take the form of administration of recombinant feline

γ-interferon or expression of feline γ-interferon within

live or disabled virus vectors.   γ-interferon has

previously been reported as being associated with adjuvancy

in animals (Heath and Playfair, 1992).   IFNγ upregulate MHC

class II expression enhancing antigen presentation, and

suppresses TH2 lymphocytes with resultant inhibition of

humoral immunity.   Through such actions an IFNγ adjuvant

can drive a vaccine-protective immunity against viruses

(Ramshaw et al, 1992).   Feline γ-interferon may also

ameliorate clinical response to live virus vaccination

which, providing protective immunity is not compromised,

could be a desirable safety feature (Andrew et al, 1991;

Ramshaw et al, 1992). Candidate live virus vectors include

poxviruses, adenovirus and herpesvirus, though the latent

capacity of the latter coupled with potential

immunopathology of cytokines may preclude cytokine

insertion in this virus.

Administration of feline γ-interferon with live feline

herpesvirus vaccines may be used to overcome mechanisms of

immune evasion or diversion specified by the virus to

provide a longer lasting protective immune response.

Embodiments of the invention will now be described by

way of example only.

EXAMPLE 1

Molecular cloning of the feline γ-Interferon cDNA

mRNA was isolated from mitogen-stimulated feline lymphocytes using the RNAgents (Trademark) Total RNA isolation system (supplied by Promega Corp.) 5µg of the mRNA was converted to cDNA using the Superscript (Trademark) system (supplied by Life Technologies).

Two oligonucleotides (see below) were designed against interspecies conserved non-coding regions of γ- interferon mRNA.

Upstream primer (I):-   5'- CTACTGATTTCAACTTCTTTGC-3'

Downstream primer (II):- 5'- CAAATATTGCAGGCAGGACAACC-3'

These oligonucleotides were then used as primers in a polymerase chain reaction (PCR) experiment to amplify the feline γ-interferon cDNA.

The PCR was performed as described by Saiki et al (1987). Ten µl of 100ng/µl template cDNA from the reverse transcribed mRNA was added to a 40µl reaction mixture containing 200µM of dATP, dCTP, of dGTP and dTTP, 50pmol of both primers (I) and (II), 1 unit of DNA Polymerase and 5µl of 10x reaction buffer. The reaction buffer contained 100mM Tris-HCl, 500mM potassium chloride, 0.01 per cent (w/v) gelatin and 1.5mM magnesium chloride, ultrapure water, TE (pH8.0). The solution was overlaid with two drops of mineral oil to prevent evaporation. To eliminate the possibility of false positives from the contamination of genomic samples with preparation of cDNA, amplification

and analysis of the products were carried out. Thirty five cylces of amplification were performed using a Perkin Elmer Cetus thermal cycler. Each cycle consisted of 1 min. at 95°C to denature the DNA, 1 min. at 50°C to anneal the primers to the template and 1 min. at 72°C for primer extension. After the last cycle a further incubation for 10mins. at 72°C was performed to allow extension of any partially completed product. On completion of the amplification, 10μl of the reaction mixture was electrophoresed through a 1.5 per cent agarose gel. The DNA was visualised by staining with ethidium bromide and exposure to ultraviolet light (320nm).

An amplified reaction product of approximately 600 bp was observed and the DNA purified and cloned into the phagemid vector PCR-Script (Trademark) sk (+) (supplied by Stratagene).

EXAMPLE 2

Sequencing of the cloned feline γ-interferon cDNA

The PCR-Script (Trademark) derived vector, containing the cloned feline γ-interferon DNA was prepared and purified. Double stranded DNA sequencing was carried out on this DNA using the T7 polymerase sequenase (Trademark) kit (supplied by USB Corp.) as per the manufacturers instructions.

The DNA sequence obtained is shown in Figure 1. The two underlined regions at the ends of the sequence correspond to the two primers (I) and (II) used to amplify the DNA. The

15

two internal underlined regions represent potential glycosylation sites.

A translation of the DNA sequence into its corresponding amino acid sequence (one-letter code) is shown below the DNA sequence.

EXAMPLE 3

Expression of recombinant feline γ-interferon in Escherichia coli.

The open reading frame (ORF) encoding the feline γ-interferon was excised from the PCR-Script (TRADEMARK) derived vector and sub-cloned into the plasmid vector pHEX (Reid, 1994 submitted) for expression.

The pHEX vector was constructed from the vector pGEX-2T (Smith and Johnson, 1988). The sequence of the glutathione S-transferase gene was deleted from pGEX-2T and a sequence which contained a consensus to the 5' coding regions of bacterial mRNA and also six histidine residues was inserted 3' to the initiating AUG (see below)

```
pHEX                                        BamHI   EcoRI
      Met Ala Lys Ile Asn His His His His His His Gly Ser Glu Phe
  tac>ATG GCT AAA ATA AAT CAT CAC CAT CAC CAT CAC GGA TCC GAA TTC
```

On expression of the cloned feline γ-interferon DNA, a polypeptide of approximately 20 kilodaltons was observed, when crude lysates of IPTG induced cultures were analysed on a 12%

16

acrylamide gel (Laemmli, 1970), followed by staining with coomasie brilliant blue dye.

EXAMPLE 4

Expression of Feline γ-interferon in baculovirus system

Feline γ-interferon cDNA, previously cloned in the pCR script ™ SK⁺ plasmid vector was excised on SstI and BamHI and cloned into similarly digested pAcCL 29-1 (Kang, 1988) which was then used to transform E.coli INVaF' (invitrogen). A stock of the feline γ-interferon containing transfer vector was then prepared using conventional techniques.

Wild type Autographa californica Nuclear Polyhedrosis virus (AcMNPV) was grown up in Sf9 cells and infectious virus DNA purified by caesium gradient and its concentration determined.

1μg of the above wild-type DNA was included in two restriction enzyme digestion reactions. Each reaction included 150μl of viral DNA, 18μl of buffer #3 (Life Sciences), 2μul of Bsu36I, 1.8μl of acetylated BSA, and 8.2μl of water to give a final volume of 180μl. The reaction was digested overnight at 370°C.

Co-Transfection of Insect Cells

Co-transfection of insect cells with linearized virus and transfer vector was achieved using the calcium phosphate co-precipitation method. To facilitate the success of this procedure, a standard calcium phosphate co-precipitation kit (Stratagene) was used. This kit employs two solutions, #1 and #2. #1 is 2.5M CaCl₂ and #2 is 2xBBS (pH 6.95) consisting of

17

50mM N,N-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid and
buffered saline, 280mM NaCl and 1.5mM Na_2HPO_4.

Dishes containing 10^6 Sf9 cells were prepared and allowed
to settle for around 2-3 hours.   Three co-transfection
reactions were set up, namely, linearized viral DNA + Transfer
vector, linearized viral DNA alone and uncut wild-type virus
alone, the latter two acting as controls. The reactions were
set up as follows: 1μg of linearized viral DNA and 5μg of
transfer vector were diluted with distilled, deionized water
to 450μl to which was added 50μl of solution #1. 500μl of
solution #2 was added slowly and the solutions mixed gently.
The mixture was allowed to incubate at room temperature for
20 minutes. After incubation the medium was removed from the
35mm dishes and 1ml of each co-transfection mixture added to
the appropriate plates. The cells were incubated for 1 hour
at room temperature. Following this incubation, a further 1ml
of TC 100 medium was added to the plates, which were then
allowed to incubate overnight at 28°C. The following day, the
medium was removed and the monolayer washed with calcium free
phosphate buffered saline. Two ml of medium was then added to
each plate and the plates allowed to incubate at 28°C for a
further 48 hours. After 48 hours the co-transfection mixtures
were harvested, filtered through a 0.45uM filter and stored at
40°C. The next step was to separate recombinant and wild-type
virus.

Separation of Parental & Recombinant Viruses by Plaque Assay
The required number of 35mm dishes were prepared, as

described, using 10^6 Sf9 cells/dish. Dilutions of the co-transfection mixtures were then made in TC100 growth medium, viz: neat, 10^{-1} 10^{-2} and 10^{-3}. The medium from the Sf9 cells was removed and $100\mu l$ of the appropriate dilution was added to the appropriate dish. The dishes were allowed to incubate at room temperature for 1 hour. Following this incubation step, the inoculum was removed and replaced with a 2ml agarose/TC100 overlay. The dishes were then incubated at 28°C for 3 days and then stained in the usual way, using neutral red and X-gal. A number of recombinant plaques were observed and one was used for subsequent experiments.

Production of Recombinant Protein

The protocol consisted of infecting cells which were in logarithmic growth (cell viability>98%) and at a concentration of 5×10^5 cells per ml. For the production of the first batch of feline γ-interferon protein, 1×10^7 Sf9 cells were used to seed 100ml of TC100 medium in a 500ml spinner culture flask. The cells were allowed to go through two doublings before being counted and subsequently infected with recombinant virus. The cells were infected when they had reached a concentration of 5×10^5 cells/ml (after 48 hours) and the infecting dose of virus used was 10 pfu/cell. The supernatant was harvested 5 days post infection and was subjected to centrifugation (1500 rpm/10mins) to remove cell debris. The product was then filtered through a 0.2uM filter and stored at -20°C. This first batch of crude interferon protein was designated DA1 and was subsequently used in the bioassays described below.

19

In addition to the production of DA1, a second batch of crude interferon was produced, designated DA2. The conditions for production were as described above but the protein was produced by the infection of High Five (invitrogen) insect cells. These cells are reported to increase protein production 10 fold.

EXAMPLE 5

Cytopathic Reduction Assay using Calicivirus Infected Feline Embryo Cells (FEA).

Two stocks of feline gamma interferon were prepared by infection of insect cells (Sf9) infected with recombinant baculovirus containing the feline gamma interferon gene. These stocks designated DA1 and DA2 were harvested on day 5 following infection with recombinant baculovirus.

A stock of feline calicivirus was prepared in FEA cells and the titre determined by plaque assay on FEA cells. In parallel, a working bank and master bank of FEA cells were grown in tissue culture and subsequently stored under liquid nitrogen.

In a 24 well plate system, FEA cells were harvested and counted from tissue culture flasks. Wells were assigned cell controls and virus controls. The cells were used to seed 24 wells at a concentration of 2×10^5 cells/well in 1ml of medium (Dulbecos MEM/10%FCS/glutamine/NaHCO3/penecillin/streptomycin and they were allowed to incubate for 24 hours at 37°C/5% CO_2. Following the incubation step the cells were examined microscopically to ensure that they had formed a sub-confluent

monolayer. Dilutions of the Sf9 medium were added to the
appropriate wells and the cells allowed to incubate at 37°C for
a further 24 hours. The control wells were incubated with
medium alone or wild-type baculovirus infected Sf9 cells.
Following incubation, the challange calicivirus was added to
the medium overlaying the cells and the cells were incubated
for a further 1 hour at 37°C/5%CO$_2$. The concentration of virus
added was sufficient to cause 100% cytophatic effect CPE, as
determined when the virus was titrated, and, in this
experiment, both 1PFU and 4PFU per cell were used. Following
the 1 hour incubation step, the medium was removed and replaced
with fresh medium. The cells were examined daily until maximum
cytopathic effect was seen in the virus controls (this was 48
hours). The medium was then removed and the cell layer stained
using crystal violet-formalin. The dye in the stained wells
was eluted and the optical density at 595nm was measured
spectrophotometrically. The results of this are represented
graphically in Figure 2 and show a sharp cut-off between
protection and non-protection, using dilutions of feline γ-
interferon.

EXAMPLE 6

Cytopathic Effect Reduction (CPER) Assay using

Vesicular Stomatitis Virus.

The VSV mutant used in this assay was designated VSVtsE2
and is derived from the New Jersey strain of VSV. This mutant
strain contains two independent mutations within the NS gene
and is incapabl of producing diseases in domestic livestock.

21

The assay procedure was essentially the same as that described for the calici virus assay. The optimum temperature for virus replication is 31°C and, initially, FEA cells were cultured at this temperature to ensure that they would still grow. The cells appeared to double normally and were subsequently infected with the VSV mutant at a rate of 0.1 PFU/cell to amplify virus stocks. The stock of virus was subsequently titrated using a standard plaque assay procedure. The 24 well plate assay system was set up as described for the calici virus assay. The only differences being that, after incubation with feline γ-interferon dilutions of DA1, the cells were challanged with VSVtsE2 at a rate of 4PFU/cell and incubation was carried out at 31°C/5%CO$_2$ in a category II laboratory.

Subsequently, the assay procedure was repeated using a 96 well format for the samples DA1 and DA2. The assay procedure for the 96 well format was essentially identical to the 24 well system. The only difference being that the cells were added to the wells at a concentration of 2x10^4 cells/well in 100μl of medium. VSV virus was added at a concentration of 4pfu/cell and the assay was carried out in triplicate. Following staining, the dye was eluted from the wells and the optical density at 595nm was measured spectrophotimetrically.

The mean optical density measurements of the 96 well VSV assays performed for DA1 and DA2 are shown graphically in Figure 3. The maximum O.D measurement shown was obtained from the cell controls and the minimum value was obtained from the virus controls. For both sampl s, DA1 and DA2, there is a

22

gradual cut off between protection and non-protection with more

points on the rectilinear portion of the graph than in the

calici virus assay. For the sample DA1 the dilution which gave

50% protection, defined in terms of dye release, was found to

be 1:8583 and, for DA2, the value was 1:1986. Thus, for sample

DA1 and using this assay protocol, the concentration of

interferon, in terms of laboratory reference units, was 8583

units/ml.

EXAMPLE 7

MHC Class II Antigen Induction Assay.

This assay was used to demonstrate that the baculovirus

derived recombinant feline interferon gamma, was able to up-

regulate MHC class II antigens on the feline continuous T cell

line, designated F422.

Initially F422 cells were grown in tissue culture and

subsequently centrifuged, washed and resuspended in complete

medium to a concentration of $3x10^5$ cells/ml. Two fold serial

dilutions of the interferon preparation DA1 were made in 12

well tissue culture plates. Wells were also assigned virus and

cell controls. 1ml of the cell suspension was added to each

well and the cells were allowed to incubate at $37°C/5\%CO_2$ for

around 48 hours.

Following incubation, the cells in each well were divided

in two to give a positive (+) and negative (-) for the

subsequent flow cytometry analysis. The "positive" cells were

labelled with MHC class II anti-cat monoclonal antibodies and

the "negative" cells were labelled with CD8 anti-cat monoclonal

23

antibodi s. The MHC class II antigen is inducible on F422 cell

lines whereas the cells are CD8 negative.

Following incubation, the cells were transferred to

labelled 5ml falcon tubes. To each tube was added 2ml of

PBS/0.1%BSA/0.01% Sodium azide to wash the cells. The tubes

were then centrifuged at 1500 rpm for 5minutes at 4°C and the

supernatant discarded. 50μl of MHC class II monoclonal

antibody was then added to the (+) cells and 50μl of CD8

monoclonal antibody was added to the (-) cells. The cells were

incubated on ice for 15 minutes. Following incubation, the

cells were washed again using 2ml PBS/BSA/azide, centrifuged

and the supernatant discarded. 50μl of FITC antibody was then

added to each tube and the tubes allowed to incubate on ice for

15 minutes. The cells were then washed with 2ml of

PBS/BSA/azide, centrifuged and the supernatant discarded.

Subsequently, cell analysis was carried out using flow

cytometry, or the cells were fixed using 1% paraformaldehyde

and analysed at a later stage.

The MHC Class II induction assay was performed on the

baculovirus interferon preparation designated DA1. This is the

same preparation that had been used in the VSV assay described

above. The results of the flow cytometry analysis is given in

Figures 4A to 4D. The gate labelled H in the figures

represents background fluorescence, while the gate labelled L

represents the percentage of cells which are demonstrating MHC

Class II up-regulation. The anti-cat MHC Class II monoclonal

antibody was used to demonstrate any changes in MHC Class II

expression on the cell line. The anti-cat CD8 monoclonal

24

antibody was us d to measure non-specific binding of mouse monoclonal antibody. The cell controls had been incubat d in the absence of interferon-gamma. The virus controls were cells which had been incubated with Sf9 cell supernatants containing wild-type PAK6 baculovirus at a 1:32 dilution. Both of these controls were labelled with anti-cat MHC class II and anti-cat CD8 monoclonal antibodies in the same way as the cells incubated with the interferon preparations. The results of the flow cytometry analysis are shown in Table 1 and in Figures 4A to 4D. The assay procedure was carried out in duplicate and the figures shown in Table 1 are mean values. All of the cells which were labelled with the anti-cat CD8 monoclonal antibody showed negligible fluorescence (see Figure 4B and Table 1).

Untreated F422 cells demonstrate low level class II expression (see Figure 4A). However, incubation of the cells with interferon dilutions clearly enhances the expression of MHC class II on these cells. This is shown by the marked shift in fluorescence as shown by comparing Figures 4A with 4C and 4D. Figure 4C shows the result of cells incubated with 1:32 dilution of interferon and Figure 4D shows the result of cells incubated with a 1:4096 dilution of interferon. At high concentrations of interferon (1:4 to 1:32), while MHC Class II was still up-regulated, the degree of up-regulation was less than for those cell incubated with lower dilutions. The MHC Class II induction assay seem to be very sensitive and up-regulation is still seen at dilutions of interferon DA1 of 1:4096.

TABLE 3

Flow cytometry of F422 cells following IFN-γ

DILUTION	% fluorescence MHC II	% fluorescence CD8
1:4	31.1	negligible
1:8	38.9	negligible
1:16	44.9	negligible
1:32	63.55	negligible
1:64	69.9	negligible
1:128	68.85	negligible
1:256	69.3	negligible
1:512	66.95	negligible
1:1024	67.6	negligible
1:2048	70.55	negligible
1:4096	71.6	negligible
cell control	1.1	0.7
virus control	1.75	0.5

26

REFERENCES

1. Andrew ME, Karupiah G, Boyle DB, Blanden RV, Mullbacher A, Ramshaw IA and Couper BEH. (1991). Effects of vaccinia virus-expressed interleukin 2 on the immune system of sublethally irradiated mice. Microbial Pathogenesis, 10, 363-371.

2. Balkwill FR and Burke F. (1989). The cytokine network. Immunol. Today, 10, 299-304.

3. Bielefeldt Ohmann HB and Babiuk LA. In vitro and systemic effects of recombinant bovine interferons on natural cell-mediated cytotoxicity in healthy and herpes-infected calves. J.Leukocyte Biol., 36, 451-456.

4. Clare JJ, Rayment FB, Ballantine SP, Sreekrishna K and Romanos MA. (1991). High-level expression of tetanus toxin fragment C in Pichis pastoris strains containing multiple tandem integrations of the gene. Biotechnology, 9, 455-460.

5. Farrar MA and Schreiber RD. (1993). The molecular cell biology of interferon-γ and its receptor. Ann. Rev. Immunol., 11, 571-611.

6. Gray PW. (1992). Interferon-γ. In: Human cytokines. Ed: Aggarwal BB and Gutterman JU. Blackwell Scientific Publications, London, pp.30-45.

7. Heath AW and Playfair JHL. (1992). Cytokines as immunological adjuvants. Vaccine, 10, 427-434.

8. Homes MA. (1993). An important milestone in feline
    immunology: feline interleukin 2. _Feline Vet.J._, 25,
    180.

9. Kang CY. (1988). Bacalovirus vectors for expression of
    foreign genes. Adv. Virus Res. 35, 177-192.

10. Laemmli UK. (1970). Cleavage of structural proteins
    during the assembly of the head of bacteriophage T4.
    Nature, 227, 680-685.

11. Mire-Sluis A. (1983). Cytokines and disease.   _Trends
    in Biotech._, 11, 74-77.

12. Radford AJ, Hodgson ALM, Rothel JS and Wood PR.   (1991).
    Cloning and sequencing of the ovine gamma- interferon
    gene. _Aus. Vet. J._, 68, 82-84.

13. Ramsay AJ, Ruby J and Bradshaw IA. (1993).   A case for
    cytokines as effector molecules in the resolution of
    virus infection. _Immunol. Today_, 14, 155-157.

14. Ramshaw IA, Ruby J and Ramsay A. (1992).   Cytokine
    expression by recombinant viruses - a new vaccine
    strategy. _Tibtech._, 10, 424-426.

15. Rinderknecht E, O'Connor BH, Rodriguez H. (1984).
    Natural human interferon-gamma. Complete amino acid
    sequence and determination of sites of glycosylation. _J
    Biol. Chem._, 259, 6790-6797.

16. Sambrook, J, Fritsch EF, Mamiatis T. (1989). Molecular
    Cloning, 2nd edition. Cold Spring Harbour Press.

17. Smith DB, Johnson KS (1988). Single step purification of
    polypeptides expressed in _Escherichia coli_ as fusion
    prot ins with glutathione S transferase. _Gene_ 67, 31-40

18. Smith GE, Summers MD and Fraser MJ. (1983). Production of human beta interferon in insect cells infected with a baculovirus vector. <u>Mol. Cell. Biol.</u>, 3 2156-2165.

19. Taya Y, Devos R, Tavernier J. Cheroutre H, Engler G and Fiers W. (1982). Cloning and structure of the human immune interferon-γ chromosomal gene. <u>EMBO J.</u>, 1,953-958.

29

CLAIMS

1.   A polynucleotide fragment encoding feline γ-interferon.

2.   A polynucleotide fragment according to claim 1 characterised in that said polynucleotide fragment encodes a polypeptide having an amino acid sequence shown in Figure 1 or a derivative thereof.

3.   A polynucleotide fragment according to claim 1 characterised in that it is a polynucleotide fragment which is substantially the same as the polynucleotide fragment shown in Figure 1 or a derivative thereof.

4.   A recombinant nucleic acid molecule comprising a polynucleotide fragment according to any one of claims 1 to 3.

5.   A recombinant nucleic acid molecule according to claim 4 characterised in that the recombinant nucleic acid molecule comprises regulatory control sequences operably linked to said polynucleotide fragment for controlling expression of said polynucleotide fragment.

6.   A recombinant nucleic acid molecule according to any one of claims 4 or 5 wherein the recombinant nucleic acid molecule is a plasmid.

7.   A recombinant nucleic acid molecule according to any one

of claims 4 or 5 wherein the recombinant nucleic acid molecule
is derived from a viral vector.

8.   A prokaryotic or eukaryotic host cell transformed, by a
polynucleotide fragment or recombinant molecule according to
any one of the preceding claims.

9.   A   recombinant   feline   γ-interferon   polypeptide   or
derivative thereof displaying substantially the same biological
activity as naturally occurring feline γ-interferon.

10.   A recombinant feline γ-interferon polypeptide as shown in
Figure 1 or derivatives thereof.

11.   An antibody immuno-reactive with a polypeptide or fragment
according to claims 9 or 10.

12.   A polynucleotide fragment according to any one of claims
1-3 for use in therapy.

13.   A recombinant nucleic acid molecule according to any of
claims 4 to 6 for use in therapy.

14.   A recombinant polypeptide or derivative thereof according
to claims 9 or 10 for use in therapy.

15.   A pharmaceutical composition comprising a polynucleotide
fragment according to any of claims 1 to 3.

31

16. A pharmaceutical composition comprising a polypeptide or derivative thereof according to claims 9 or 10.

CTACTGATTTCAACTTCTTGGCCTAACTCTCCGAAACGATGAATTACACAAGTTTATT
. L S E T M N Y T S F I

TTCGCTTTCCAGCTTTGCATAATTTGTGTTCTTCTGGTTATTACTGTCAGGCCATGTTT
F A F Q L C I L C S S G Y Y C Q A M F

TTTAAAGAAATAGAAGAGCTAATGGGATATTTAATGCAAGTAATCCAGATGTAGCAGAT
F K E I E E L M G Y F N A S N P D V A D

GGTGGGTCGCTTTCGTAGACATTTGAAGAACTGGAAAGAGGAGAGTGATAAAACAATA
G G S L F V D I L K N W K E E S D K T I

ATTCAAAGCCAAATTGTCTCCTTCTACCTGAAAATGTTTGAAAACCTGAAAGATGATGAC
I Q S Q I V S F Y L K M F E N L K D D D

CAGCGGCATTCAAAGGAGGCATGGACCACCATCAAGGAAGACATGCTTGATAAGTTGTTAAAT
Q R I Q R S M D T I K E D M L D K L L N

ACCAGCTCCAGTAAACGGGATGACTTCCTCAAGCTGATTCAAATCCCTGTGAATGATCTG
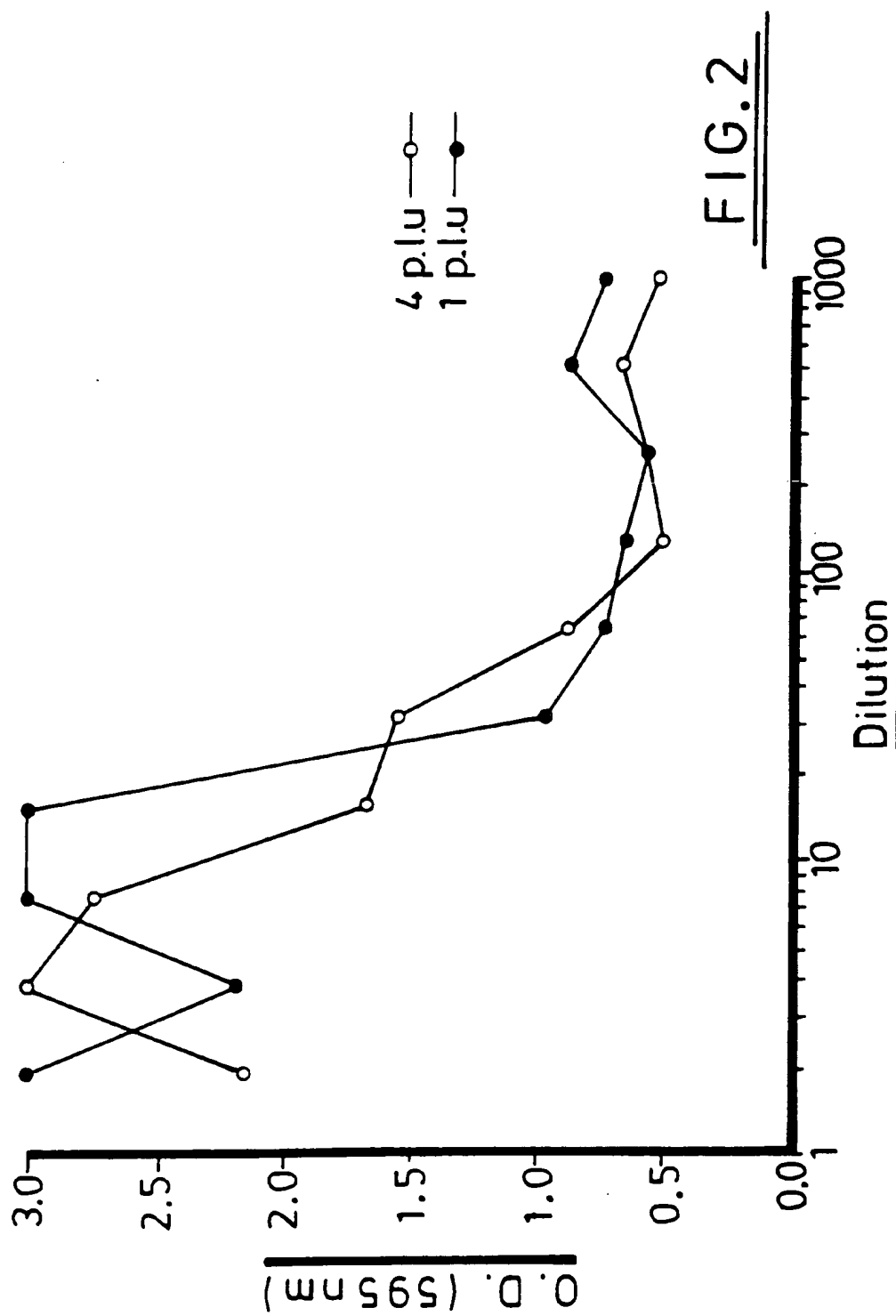T S S S K R D D F L K L I Q I P V N D L

CAGGTCCAGCGCAAAGCAATAAATGAACTCTTCAAAGTGATGAATGATCTCTCACCAAGA
Q V Q R K A I N E L F K V M N D L S P R

TCTAACCTGAGGAAGCGGAAAAGGAGCCAGAATCTGTTCGAGGCCGTAGAGCATCGAAA
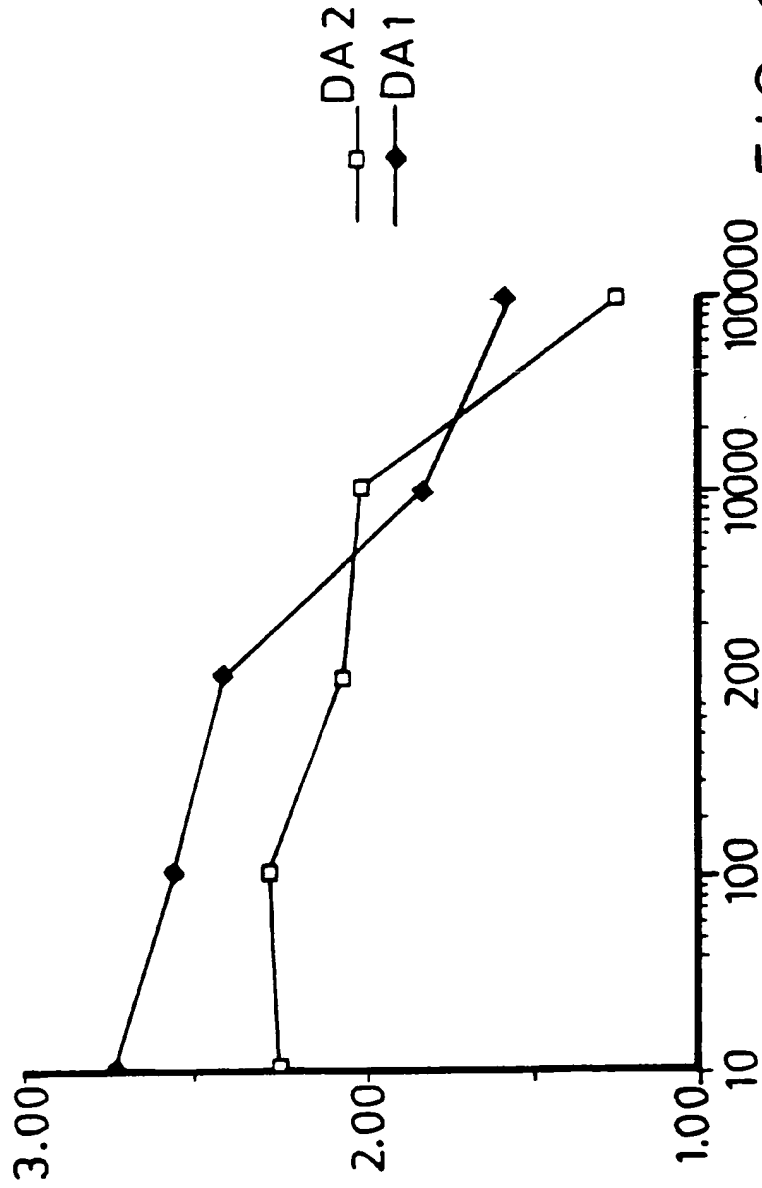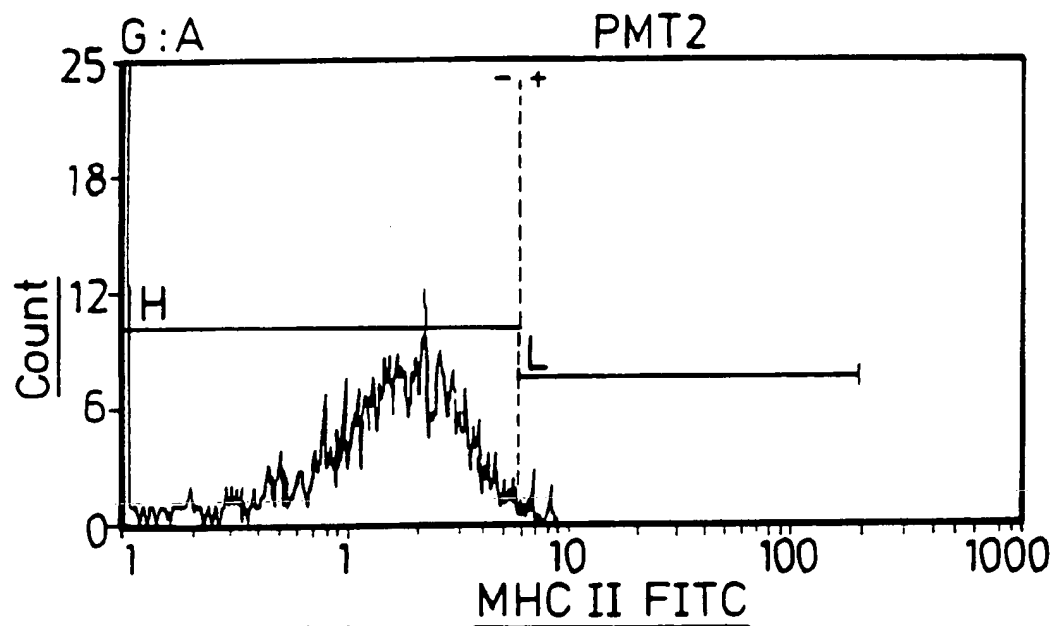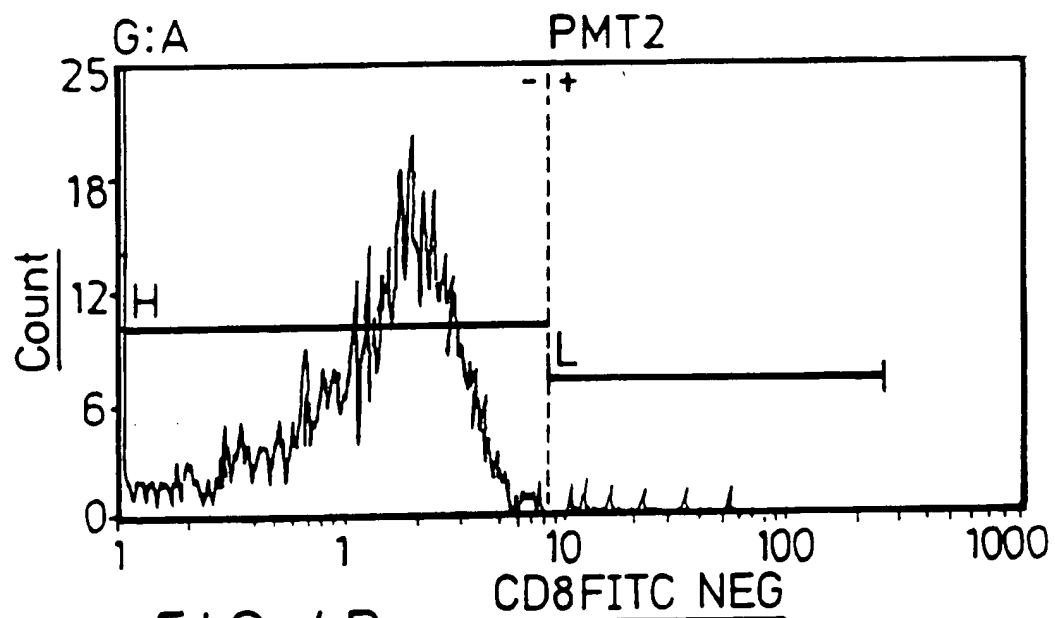S N L R K R K R S Q N L F R G R R A S K

TAATGGTTGTCCTGCCTGCAATATTTG
*

FIG. 1

FIG.2

FIG.3

FIG.4A

FIG.4B

FIG.4C

FIG.4D